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DETERMINATION OF HEPARIN IN BLOOD

C. H. Hemker and S. Béguin

INTRODUCTION

Probably the most important antithrombotic drug in use at the moment is heparin (1-10). Actually the annual sale in the Netherlands is $6 \cdot 10^{10}$ U, that is 2000 U per head of the population. Its administration by injection and the fact that the prescribing doctors are well aware of the inherent risk of bleeding (11) make that it is not likely to be used inadvertently. The consumption in the Netherlands is probably the minimum that can be used in any medical community that takes the prevention and treatment of thrombosis seriously because especially in this country the use of the only serious alternative - i.e. oral anticoagulation - is very well organized and hence very common (12,13).

In recent years we have witnessed the advent of low molecular weight heparins (LMWH), there are indications that they may be more convenient drugs than unfractionated heparin (UFH) is (14-23). This promises that heparin usage will still increase. In fact the discussion that is going on on LMWH already seems to bring about a rise in the use of UFH.

This situation makes that the question of how to determine heparin in the plasma of a patient becomes more and more urgent. Logically another question should precede the question of *how* to determine heparin, and that is *why* to determine heparin. Is it necessary to determine heparin levels (or a related parameter) or will giving standard doses do the job? That fundamental question can be answered only from controlled clinical trials and for such trials an adequate heparin test must be available. This is a circular argument. Therefore the question of what makes a good heparin test may well be the one to start with.

CURRENT TYPES OF HEPARIN TESTS

All current determinations of heparin are based on its influence on the blood coagulation process i.e. its enhancing effect on the inactivation of activated clotting factors by antithrombin III (AT III). Several other actions of heparin have been described however. It binds to the vessel wall (24-27), influences fibrinolysis (28-32), causes the release of lipoprotein lipase (33-38) etc. We keep thinking that its main therapeutic action is on blood coagulation for a simple reason: vitamin K antagonists also have a large spectrum of actions apart from that on coagulation. Yet coagulation is the only one that it has in common with heparin. It seems more likely than not that it is indeed this common denominator that has the antithrombotic effect. Until proof of the contrary we therefore

think that a test of the coagulation system is the most adequate to reflect the therapeutic action of heparin.

What are the current coagulation based tests of heparin activity? They come in several kinds:

1. **Thrombin time (TT)** (39-42). These tests are based upon the clotting of plasma by a fixed amount of added thrombin.
2. **Activated partial thromboplastin time (aPTT)** (41-50). Based upon starting coagulation in the patient's plasma via the intrinsic pathway.
3. **Heptest** (39,51,52). A clotting time started with activated factor X among other things.
4. **Anti-thrombin tests** (39, 53-59, 62). An amount of thrombin is added and the test measures the amount that remains active after a fixed incubation time.
5. **Anti-factor Xa tests** (39,55-62). Like 4, but now with factor Xa rather than with thrombin.
6. **Neutralisation tests** (63, 64).

Under 6 we group a number of more specific and more precise tests that are not in routine use, often because they are relatively time consuming or require techniques not readily available in the clinical laboratory. The neutralization tests, based upon titration of the heparin in the sample with a heparin neutraliser such as protamine are mainly used for determining precisely the high heparin concentrations met in extracorporeal circulation. These will not be discussed further because we focus on tests for heparin treatment and prevention here.

Tests 1, 2 and 3 are clotting tests; 4 and 5 are spectrophotometric tests based on the use of thrombin- or factor Xa-specific chromogenic substrates. Tests 1 and 4 measure the anti-thrombin effect, tests 3 and 5 the anti-factor Xa effect. The way in which heparin influences the aPTT has until recently remained relatively unclear. Especially the reason why the aPTT is sensitive to heparin whereas the PT (Quick-time) is not, remained obscure. We demonstrated that the aPTT is primarily dependent upon the time necessary for trace amounts of thrombin to activate so much factor VIII that a burst of thrombin formation ensues (65,66). This positive feedback effect of thrombin on its own formation is the main feature of the kinetics of the aPTT. Heparin prolongs the aPTT by enhancing the action of AT III on the trace amounts of thrombin that act as a feedback activator. Once the burst of massive thrombin generation occurs coagulation follows immediately, largely independent of the *amount* of thrombin that is formed. In the PT the feedback activation of factor VIII does not play a role, as would be expected, but, possibly because of the large excess of factor Xa and the availability of factor V (not bound to von Willebrand factor), the feedback of thrombin on factor V does not play a role either. Heparin will decrease the peak amount of thrombin formed in both aPTT and PT. That the PT is insensitive to heparin illustrates the fact that decreasing the peak *amount* does not influence the *clotting time* (see later).

The Hepptest is a kind of aPTT that is started by the addition of activated factor X to the sample. If factor Xa is rapidly broken down, as in the presence of heparin (either UFH or LMWH) this test will prolong. On the other hand it also is sensitive to thrombin inactivation. The first effect is more important though and the Hepptest therefore may be regarded as "the poor man's anti Xa" (64).

For several years it has been thought that the antithrombin action of heparin was responsible for bleeding whereas the anti-Xa action was thought to reflect antithrombotic power (67-69). More recent research very much doubts whether this is the case (15, 70-71). In fact it could be demonstrated that UFH in the concentration range used for prophylaxis or treatment of thrombosis has only a very small effect on the conversion velocity of prothrombin in clotting plasma (65, 66, 72, 73) while its antithrombotic action is not questioned. On the other hand a trial on a LMWH that had a high anti-Xa action had to be stopped because of the many bleeding complications in the treated group (21).

PLATELETS AND HEPARIN

Before discussing this point any further it is necessary to consider the important effect of blood platelets on heparin. Whereas the inverse, the effect of heparin on blood platelets is often the subject of discussion, without for that matter leading to a very clear picture (75-84), the effect of platelets on heparin is straightforward and clearcut (65, 85). Blood platelets, when activated, shed heparin neutralizing material such as platelet factor 4 (PF4), a protein with strong antiheparin properties (86-92). The 3.10^8 pL of normal platelet rich plasma (PRP) can neutralize about 0.3 U/ml of UFH. Normal venepuncture will always cause some platelet activation, especially when carried out under routine clinical conditions and even more so when vacuum tubes are used. This means that important amounts of PF4 may be shed so that between 0 and 0.4 U/ml of heparin will escape attention! Collecting blood on a platelet inhibitor is a partial solution to this problem. Unless this is done any exact heparin determination is illusory and 0.18 \pm 0.07 U/ml will be neutralised in the sample (93). Even when collecting on platelet inhibitor the difference between heparin in a patient's sample and spiked heparin remains, be it to a lesser extent (94,95).

The very fact that platelet activation is an important event in haemostasis and thrombosis makes that the PF4 susceptibility of a heparin will be one of the factors that determine its *in vivo* action. If thrombin generation in PRP is triggered by small amounts of tissue thromboplastin, a test system is obtained that is one step nearer to the *in vivo* situation than tests in platelet poor plasma (PPP) are. Under these circumstances a burst of thrombin formation is observed about five minutes after starting the reaction. In the presence of UFH this burst is dose-dependently postponed but the amount of thrombin formed is not diminished. This is because trace amounts of thrombin that are generated during the lag-phase activate the platelets. Once the platelets are activated they provide

procoagulant phospholipids and factor V which allow a massive increase of thrombin formation. The situation is completely comparable to the feedback activation of factor VIII discussed above but even more outspoken. The amount of thrombin available during the lagphase is less in the presence of heparin and hence the lagphase will prolong. Once the platelets are activated they shed so much PF4 that the heparin is neutralized and the thrombin then formed is not or hardly inhibited at heparin concentrations up to 0.4 U/ml. None of the current tests takes this effect into account. This is the more regrettable because here an important difference between UFH and LMWH shows up. LMW Heparins are much less susceptible to PF4 inactivation than UFH and each of them to a different extent (96,97).

HOW TO CHOOSE A HEPARIN TEST?

For a good heparin test there is one minimal requirement: it should indicate the amount of active material in the circulation of the patient. Indeed there need not even be a relation between the property measured and the property that carries the pharmacological effect, a correlation will do. If heparins would strongly adsorb at a convenient wavelength or carry detectable radioactivity, then these properties could be used for measuring heparin concentrations in plasma. Even if the molecules that carry the signal would not be the same as those carrying the pharmacological effect.

On the other hand one dreams of the ideal test, which does not inform us about the amount of material in the blood but about the effect of that material on the relevant properties of the blood. In other words: the test that would not be a concentration-indicator but a true model of the therapeutic efficacy.

It looks as if, in the heparin field, we do have an acceptable test of the first type, whereas we are far from having one of the second type. This is all the more regrettable because heparins are notoriously heterogeneous. This makes that any property that we measure and that has not been proven to indicate therapeutic efficacy directly, may fool us as soon as the proportion of the indicator property to the effector property changes.

There are at this moment good reasons to believe that anti-factor Xa activity is an acceptable indicator for concentrations of circulating heparin. In many clinical studies it has been shown to correlate with the effect, at least when LMWHs are compared and within the rather broad limits that are set by clinical dose finding studies (98,104). From animal studies one has good grounds to doubt whether it also holds for a purely anti-factor Xa heparin like the Choay pentasaccharide (105).

Particularly interesting is the study of Cadroy in baboons that presents a model of the arterial platelet thrombus and that compares the dose-effect curves of UFH and of the very low molecular weight heparin CY222 in that model (106). The results of this study have been interpreted as to indicate the practical usefulness of the anti-thrombin test (107). This seems indeed a sound conclusion. Concluding however on basis of these tests that anti-thrombin measurements or even weight would be less useful seems precarious.

The matter can be conveniently discussed in terms of IC₅₀ concentrations (108). When anti-factor Xa activity is used to compare the IC₅₀'s of both heparins, then they appear to be much closer than when they are expressed in terms of either weight or anti-thrombin activity (table 1). This is the core of reasoning of ref. (107).

Table 1. Comparison of IC₅₀ values of two different heparins.

	UFH	CY222	Ratio
IC ₅₀ (μg/mL)	6.25	33	0.2
Anti Xa (U/mL)	1	1.4	0.7
Anti IIa(U/mL)	1	0.15	6.7
High affinity mat.(%)	36	10,	
IC ₅₀ H.A.mat (mg/mL)	2.25	3.3	0.7
Decay constant at IC ₅₀			
Factor Xa	37.5	26.4	1.4
Factor IIa	81.2	29.7	2.7

This certainly proves the practicability of the anti-factor Xa activity test. Does it however settle the argument in disfavour of either weight or anti-thrombin activity tests? Let us discuss weight first. What substance should be weighed? Obviously the active substance. Even though it has been described that non-AT III binding heparins contribute to the antithrombotic activity (109), one is rather tempted to think that the amount of AT III binding material is the important thing. We determined, by fluorescence-titration of pure AT III the percentage high affinity material in UFH and CY 222. If the IC₅₀s are calculated in weight of AT III binding material, it appears that the difference between UFH and CY222 is mainly due to a difference in content of high affinity material (table 1). Thus the apparently strong case against weight as a basis of comparison seems to weaken. Below we will argue that also the anti-thrombin activity may not be as easily dismissed as would be thought on basis of a quick interpretation of Cadroy's results.

Nevertheless one must admit that the presently available anti-thrombin tests are less sensitive than the anti-test, especially in the case of LMWHs. It remains an interesting question whether its sensitivity could be increased by rigorous prevention of platelet activation in the samples.

The aPTT test measures principally the effect of heparin on the feedback activation of factor VIII by thrombin and on factor IXa inactivation during the lag phase of thrombin formation (65,66,110). It is an indirect test of antithrombin action that is more sensitive than the direct test is because it takes advantage of the amplifying effect of the feedback mechanism. The main drawback is the large individual variation. It has been shown that spiking individual patient plasmas with the same amount of heparin results in very different prolongation ratios of the aPTT. This is probably due to the fact that the test is sensitive to the levels of all clotting factors, all heparin binding proteins and also to pathological inhibitors (111-116). Also different types of the test (as to contact activator used and phospholipid preparation) will give different outcomes.

We conclude that the anti-factor Xa activity test seems to fulfill the minimal requirements for a heparin determination. In comparing it to other tests, it must be said in its favour, that it seems to be more sensitive and less subject to inter patient variation than the trombin time and the aPTT, especially with LMWHs. The Hepptest seems to hold an intermediary position here.

HOW TO FIND A MODEL FOR CLINICAL EFFICACY?

After having argued that the anti-factor Xa activity test at this moment is probably the best available practical shortcut to heparin measurement, we will now show that it can by no means be accepted as a model for clinical efficacy. This may seem a superfluous remark because it never has been claimed that it would be. It is well known however that any test that is in general use gets a respectability of its own that might make it slip stealthily in the position of the accepted standard (117).

We found a method that enabled us to measure the velocity of prothrombin conversion in clotting plasma, independant of the simultaneous thrombin inactivation. Surprisingly CY222 and other substances that show a high conventional anti-factor Xa activity, do not show a parallel inhibition of prothrombin conversion in the extrinsic system. This is an unexpected result because the only physiological effect of factor Xa is prothrombin conversion. The reason is that factor Xa is produced during coagulation in such excess that it can be inhibited quite markedly before any effect on prothrombinase activity can be observed. In the intrinsic system heparin does inhibit prothrombin conversion, but this must be attributed to a secondary effect on the feedback activation of factor VIII by thrombin and to inhibition of factor IXa (64,65,110).

The main effect of heparins, be it fractionated or unfractionated, is inactivation of thrombin. The only exception are very low molecular weight products like pentasaccharide and certain subfractions of the usual LMWHs (64,96,97).

If indeed anti-thrombin activity is the main action, then why do the IC50s of that property not compare? (table 1). The reason is probably that the anti-thrombin activity and the anti-factor Xa activity as they are routinely measured bear only a distant relationship to the same properties in clotting plasma. In the study of Cadroy the anti-thrombin activity was measured by the aPTT and by the TT. These can not be accepted as exact quantitative estimations of the anti-thrombin effect (see above). Neither is the anti-factor Xa activity as measured routinely a good indicator of what happens to factor Xa in plasma. It has been shown that the decay of factor Xa in plasma varies both with the species from which the factor Xa is prepared and with the concentration of Ca^{++} ; moreover these variations are not the same with different types of heparin. It therefore will make an enormous difference whether bovine factor Xa decay is measured in the absence of Ca^{++} , as in the anti-Xa test, or human Xa in the presence of Ca^{++} , as in vivo.

We measured the specific decay constants of standard UFH and of CY222 in human plasma with human factor Xa and at physiological Ca^{++} concentration. Then we calcu-

lated what the effect of the IC₅₀s of Cadroy would be in PPP on the decay of thrombin and factor Xa. We see that the almost tenfold difference between anti-factor Xa activity and anti-thrombin activity of the original data tend to diminish to a bare twofold (table 1). We are conscious that we are applying data from human plasma to the *in vivo* situation in baboons, but it is the best approximation that we can offer for the moment.

THE SEARCH FOR IDEAL TEST

We must conclude that the ideal test for anticoagulant treatment does not yet exist. We therefore should try and make one. Such a test should meet a number of stiff demands, it should:

- a.* Be equally sensitive to comparable therapeutic levels of oral anticoagulation, and of treatment by heparin or heparinlike drugs.
- b.* Be sensitive to the inhibition of factor Xa (or any other clotting factor (notably factor Xa)) only insofar as this factor influences thrombin generation.
- c.* Be either not hampered by platelet activation (PPPtest) or take physiological platelet activation into account (PRPtest).

There is only one procedure that, as far as we can see, approaches these requirements. It has been in practical use for many decades and is often tacitly assumed to be the golden standard for effects on the coagulation system. We mean the thrombin generation test (TGT). It is indeed influenced by all anticoagulation procedures. It reflects inhibition of individual clotting factors, but not proportionally to their percentual variation, except for prothrombin (117). It can be carried out equally well in PRP as in PPP.

The thrombin generation curve of course has a mayor drawback: it takes an experienced laboratory, much time and a lot of reagent. This makes that until now it has practically been used for research purposes only.

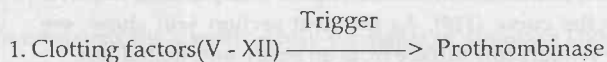
In order to develop a routine test on this basis one should first decide what type of value one wants to measure. A classical TGT shows a number of different features. One can measure the lagtime of thrombin formation, the peak value of thrombin obtained or the surface under the curve. The lagtime is equivalent to a clotting time, because clotting is invariably seen at the beginning of the thrombin burst. In our experience the surface and the peakvalue correlate strongly. There is a theoretical argument to favour the surface under the TGT as an essential variable: thrombin is an enzyme. One nanomolar for ten minutes will in principle have the same effect as ten nanomolar for one minute. This means that the product of time and thrombin concentration is important, which is equivalent to the surface under the curve (118). As the next section will show, we developed a method that directly renders this surface from an easily automated test.

A CANDIDATE: THE THROMBIN POTENTIAL AND HOW TO MEASURE IT

As discussed above, it is a common property of all antithrombotic drugs that they diminish the amount of thrombin generated in clotting plasma, either because there are less clotting factors present (oral anticoagulation) or because the thrombin formed disappears faster (heparins etc.). Whether heparins also inhibit (the formation of) prothrombinase and inhibit thrombin formation also in that way is of no importance for the present purpose. If they do this effect is also measured by the procedure that we are going to discuss. Under antithrombotic treatment as described above the form of the thrombin generation curve changes in one or more of the following respects: it starts later, i.e. the lagphase before explosive thrombin formation occurs is longer, the peak is lower or the decay is faster so that the thrombin present is inactivated earlier. It has been shown that a lower generation (oral anticoagulation) as well as a faster decay (heparin treatment) cause efficacious antithrombotic treatment. Both result in a smaller surface under the thrombin generation curve. This surface we define as the endogenous thrombin potential (ETP) of the plasma. We here present the outlines of a method to determine the ETP and we think that the ETP thus measured can, among other things, serve to measure the effect of any known anticoagulant drug: heparins and heparin likes, hirudin and oral anticoagulation.

In the assay that we propose a test sample of blood, platelet rich plasma, platelet poor plasma or defibrinated plasma is added to a solution containing a trigger of the coagulation process, a source of Ca^{++} ions, a preparation of a natural antithrombin and a substrate of thrombin. The natural antithrombin may be antithrombin III or heparin cofactor II or both. They serve the purpose of rendering the test less sensitive to variations in the concentration of these inhibitors in the test sample and to minimise the side reaction with other antithrombins such as α_2 -Macroglobulin. By selecting either ATIII or HCII the test can be made specifically sensitive to drugs of the heparin, resp. of the dermatan sulfate type. It is not even excluded that under certain circumstances it may not be absolutely necessary to add extra inhibitors to the reaction medium. What is essential to the present test is that a substrate is used that is not exhausted during the lifetime of the thrombin that generates and disappears in the clotting plasma. The mixture with the test sample is incubated during a specified time under specified conditions. Then the amount of thrombin substrate is read directly or, in opaque solutions the reaction is stopped by adding a thrombin inhibitor and the mixture is treated, e.g. by centrifugation in order to allow determination of the amount of product formed or the amount of substrate that has disappeared. Also is it possible in optically clear solutions to monitor spectrophotometrically the amount of product formed. This is of particular interest because the first derivative of that curve will represent the course of thrombin generation in the sample.

The following reactions further illustrate the present method:



- Prothrombinase
2. Prothrombin —————> Thrombin
3. Thrombin + Antithrombins ———> Inactive complexes
- Thrombin
4. Indicator Agent —————> Signal Molecule

Reactions 2 and 3 go to completion so that thrombin is only transitorily present in the reaction mixture. During its presence it partakes in reaction 4, so that the extent of the conversion of the indicator agent reflects the time during which- and the amount with which, thrombin has catalysed that reaction. It is essential that the indicator agent is not exhausted before the thrombin has disappeared. The reaction velocity should be proportional to the concentration of thrombin at any moment, i.e. the concentration of substrate has to be several times higher than K_m .

In a test set up according to this principle a variety of different triggers may be used: thromboplastins of any origin for investigating the ETP of the extrinsic pathway, contact activators etc.

In performing the assay a great variation of protein concentrations, incubation times, coagulation triggers, reagent concentrations and temperatures may be used. These parameters may be varied according to the special purpose that the determination of the ETP may serve e.g. determination of low molecular weight heparins, monitoring of oral anticoagulation, detection of hyper-coagulable states etc.

The end level of substrate conversion is not necessarily completely fixed under all circumstances, because the complex of thrombin to α_2 -Macroglobulin has a persistent amidolytic activity. It may therefore be necessary to measure the optical density at a fixed moment after starting the reaction, or, if the reaction mixture is not optically clear, such as with the use of whole blood, to stop the reaction with a quenching solution and subject the reaction mixture to manipulations (such as centrifugation or precipitation) that allow measurement of the amount of the signal molecule present. Any chemical that will stop the action of thrombin on the thrombin substrate can in principle serve this purpose. Benzamidine, hirudin and α hNAPAP are obvious candidates.

The indicator agent can be any molecule capable of reacting with thrombin. In such reaction products should be formed which produce a measurable signal. P-nitroaniline, easily detectable fotometrically at 405 nm is an obvious choice but other indicator agents may be used, such as those producing fluorescent or electrochemical signals.

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